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Dioxin Exposure Blocks Lactation Through a Direct Effect on Mammary Epithelial Cells Mediated by the Aryl Hydrocarbon Receptor Repressor

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Running title: *Dioxin directly blocks mammary lactation*

ABSTRACT

In mammals, lactation is a rich source of nutrients and antibodies for newborn animals. However, millions of mothers each year experience an inability to breastfeed. Exposure to several environmental toxicants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been strongly implicated in impaired mammary differentiation and lactation. TCDD and related polyhalogenated aromatic hydrocarbons are widespread industrial pollutants that activate the aryl hydrocarbon receptor (AHR). Despite many epidemiological and animal studies, the molecular mechanism through which AHR signaling blocks lactation remains unclear. We employed *in vitro* models of mammary differentiation to recapitulate lactogenesis in the presence of toxicants. We demonstrate AHR agonists directly block milk production in isolated mammary epithelial cells. Moreover, we define a novel role for the aryl hydrocarbon receptor repressor (AHRR) in mediating this response. Our mechanistic studies suggest AHRR is sufficient to block transcription of the milk gene *β-casein*. Since TCDD is a prevalent environmental pollutant that affects women worldwide, our results have important public health implications for newborn nutrition.

Keywords: lactation, AHR, TCDD, AHRR, ARNT, mammary gland

Lactation is a critical biological process in mammals that provides both nutritional and immune support for offspring. However, an estimated 3-6 million human mothers worldwide suffer from impaired lactation each year (Lew *et al.*, 2009). Several factors contribute to milk production and secretion, but a growing number of studies suggest certain environmental toxicants negatively impact the ability of women to initiate and sustain breastfeeding (Neville and Walsh, 1995). For example, maternal exposure to pesticides has been associated with shortened duration of lactation in both the United States (Rogan *et al.*, 1987) and Mexico (Gladen and Rogan, 1995).

One specific xenobiotic known to affect lactation is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is a byproduct of natural processes, such as volcanic eruptions and forest fires, and industrial processes, including smelting, waste incineration, pesticide production, and combustion (Wong *et al.*, 2012). Once produced, TCDD persists in the environment and contaminates air, soil, and food sources (Larsen, 2006; Travis and Hattemer-Frey, 1991). Although inhalation and skin exposure occur, ingestion of contaminated food sources is the primary route of exposure for humans (Carpenter, 2006). With an average half-life between 7 and 8 years in humans (Geyer *et al.*, 2002), TCDD is a highly stable chemical that bioaccumulates in fat tissue. Thus, TCDD exposure amasses and persists overtime (Consonni *et al.*, 2012).

Previous studies have demonstrated TCDD exerts its toxicity through activation of the aryl hydrocarbon receptor (AHR) (Fernandez-Salguero *et al.*, 1996). As a ligand activated transcription factor, AHR is restricted to the cytoplasm in its unbound state. Once activated, AHR translocates to the nucleus and forms a transcriptionally active complex with the AHR nuclear translocator (ARNT) to alter expression of specific target genes (Tijet *et al.*, 2006).

Although the identity of endogenous AHR activators remains controversial, several exogenous chemicals have been shown to target AHR (Denison and Nagy, 2003). In particular, we previously identified a 1,2,4-*bis*-aryloxadiazole, referred to as 1023, as a novel AHR agonist (Basham *et al.*, 2014; Basham *et al.*, 2013).

An association between AHR activation and changes in milk production has been observed in animal studies. Specifically, pregnant mice exposed to TCDD *in vivo* produced lower levels of the milk proteins β-casein (Collins *et al.*, 2009) and whey acidic protein (WAP) (Vorderstrasse *et al.*, 2004), and were unable to nutritionally support their offspring (Vorderstrasse *et al.*, 2004). Moreover, exposure of pregnant rats to TCDD led to severe defects in mammary gland differentiation and decreased pup size following lactation (Badesha *et al.*, 1995; Fenton *et al.*, 2002), suggesting TCDD exposure impaired functional development of the mammary gland. Together with epidemiological studies in humans, these observations support a strong link between AHR activation and diminished milk production.

Despite these studies, the molecular mechanism through which AHR signaling blocks milk production remains unclear. Moreover, reciprocal transplant studies with AHR null mammary glands implicate both indirect, systemic effects and direct, cellular consequences of AHR signaling on alveolar differentiation (Lew *et al.*, 2011). Indirectly, TCDD disrupts endocrine function by altering estrogen-mediated signaling (Matthews and Gustafsson, 2006; Safe *et al.*, 1998). However, changes in circulating estradiol, progesterone, or prolactin levels were not observed in pregnant mice after *in vivo* TCDD exposure (Vorderstrasse *et al.*, 2004), which suggests mammary tissue may respond directly to AHR agonists. Moreover, explant studies, where mammary glands were cultured *ex vivo* under hormonal stimulation with TCDD, showed decreased lobuloalveolar structures (Hushka *et al.*, 1998). Together, these studies

suggest AHR signaling contributes to impaired lactogenesis by directly targeting mammary tissue.

Based on these initial observations, we hypothesized that the epithelium had a significant role in mediating the response to TCDD. Thus, we aimed to identify direct epithelial mechanisms through which AHR activation blocks β-casein production. Using *in vitro* models of mammary morphogenesis and differentiation, we show that both environmental toxicants (TCDD) and novel AHR agonists (1023) block lactogenesis directly in mammary epithelial cells. Furthermore, we identify a new role for the aryl hydrocarbon receptor repressor (AHRR) in mediating this response. Our results support a model in which AHRR induction promotes formation of AHRR/ARNT heterodimers, which transcriptionally inhibit β-casein production.

MATERIALS AND METHODS

Mice. Mice were maintained following protocols reviewed and approved by the University of Utah Institutional Animal Care and Use Committee.

Isolation of primary MECs. Organoids from the fourth inguinal mammary gland were isolated from virgin (8-12-week-old) female mice and processed to single epithelial cells as previously described (Basham *et al.*, 2013).

Microarray data. Data previously generated (Basham *et al.*, 2013) were analyzed using GeneSifter software (Geospiza Inc, Seattle, WA). Microarray data analyzed for this publication can be obtained from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) using accession number GSE39249.

Chemical compounds. TCDD (Cambridge Isotopes Laboratories, Inc., Andover, MA) was obtained as a DMSO stock solution and used at a final concentration of 10 nM, which is

within the range of exposures (0.1 nM - 100 nM) previously reported to be environmentally and pharmacologically relevant (Birnbaum and DeVito, 1995). 1023 was synthesized as previously described (Basham *et al.*, 2013) and dissolved in DMSO at a stock concentration of 10 mM. 1023 was used at a final concentration of 10 μM, which was selected based on a previous dose response analysis in mammary epithelial cells (Basham *et al.*, 2013), where 1023 exhibited an EC_{50} of 1.2 +/- 0.050 μM. Based on these results, 10 μM was the minimum concentration required to achieve maximal effect.

HC11 cell culture and induction with lactogenic hormones. HC11 cells were maintained at 37°C with 5% CO2 in HC11 culture media (RPMI (HyClone, Logan, UT), 10% FBS (HyClone, Logan, UT), ITS-X (Invitrogen, Carlsbad, CA), 1X penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), and 10 ng/ml murine epidermal growth factor (EGF) (BD Biosciences, San Jose, CA)). For induction with lactogenic hormones, $1x10^6$ cells per well were seeded in 6-well tissue culture plates. Two day confluent cultures were washed twice with EGF-free HC11 culture media and grown for 96 additional hours in EGF-free HC11 culture media containing 5 μg/ml ovine prolactin (Sigma, St. Louis, MO) and 1 μM dexamethasone (Sigma, St. Louis, MO). For compound treatments, FBS was reduced to 2% 12 hours after seeding and cells were grown with 0.1% DMSO, 10 μM 1023, or 10 nM TCDD as indicated. Throughout each assay, media was replaced every 48 hours.

AHR nuclear localization assay. In 12-well tissue culture plates, 60,000 HC11 cells stably expressing pEiZ-HA-AHR were seeded onto lysine-coated glass coverslips. Cells were seeded in HC11 culture media containing 10% charcoal-stripped FBS (CSFBS) (Sigma, St. Louis, MO). The media was replaced 12 hours later with HC11 culture media containing 2% CSFBS. Cells were dosed 36 hours post-plating with 0.1% DMSO (Sigma, St. Louis, MO), 10

μM 1023, or 10 nM TCDD. Samples were stained 24 hours after dosing. For each condition, a minimum of 100 cells per sample was scored.

Immunofluorescence. For HA (4 μg/mL, Abcam, Cambridge, England, #ab9110) staining, HC11 cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT), washed once with 50 nM ammonium chloride (Sigma, St. Louis, MO), and permeablized with 0.2% triton X-100 in PBS for 8 minutes at RT. Cells were washed once in 1% BSA in PBS and blocked with 5% BSA and 1% normal goat serum in PBS for 10 minutes. Following block, samples were incubated with primary antibody diluted in 1% BSA in PBS for 1 hour at RT. After primary antibody incubations, samples were washed three times with PBS and incubated with secondary antibody (1:1000 in 1% BSA in PBS Invitrogen, Carlsbad, CA, Alexa series) for 1 hour at RT. Nuclei were stained with DAPI (50 ng/mL, Molecular Probes, Eugene, OR) for 5 minutes at RT. Coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen, Carlsbad, CA).

Microscopy. Imaging was performed using an Olympus IX81-ZDC microscope with an ORCAER CCD camera and Slidebook 5.0.0.24 software (Intelligent Imaging Innovations, Inc, Denver, CO). A 60X Plan oil objective lens was used for representative images and a 40x U-Plan objective lens was used for images for quantification.

Western blot. HC11 cells were washed twice and scraped in 1 ml cold PBS. Cells were pelleted at 1250 x g for 5 minutes, lysed in RIPA buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCL, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM DTT, 1X protease/phosphatase inhibitor cocktail), and sonicated for 20 seconds. All processing steps occurred at 4°C or on ice. Whole cell lysate (50 μg) was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore, Billerica, MA) for detection of β-casein (1:100, Santa Cruz, Dallas, TX, #sc-17969), α-tubulin (1:1000, Sigma, St. Louis,

MO, #T6199), hemagglutinin (HA) (1:500, Covance, Princeton, NJ, #MMS-101R), or ARNT (1:500, BD Biosciences, San Jose, CA, #611078). Integrated intensity values were measured on a LI-COR Odyssey Scanner (Lincoln, NE) with median background subtraction and normalized to α-tubulin levels.

RNA extraction, cDNA synthesis, and real-time PCR. RNA was isolated, converted to cDNA, and used for real-time PCR as previously described (Basham *et al.*, 2013). Primer sequences were as follows: *β-actin* forward (5'-GGCTGTGCTGTCCCTGTATG-3'), *β-actin* reverse (5'-CAAGAAGGAAGGCTGGAAAA-3'), *β-casein* forward (5'-CAATCCCGTCCCAC AAAA-3'), *β-casein* reverse (5'-TCCAGTTTCAGTCAGTTCAAAAA-3'), *α-casein* forward (5'-CAGAAGTGCTCTCTGTCTGTTCA-3'), *α-casein* reverse (5'-AGCATAGCAGCAGTGAGGAAA-3'), *AHR* forward (5'-CTTTGCTGAACTCGGCTTGC-3'), *AHR* reverse (5'-TTGCTGGGGGCACACCATCT-3'), *Cyp1A1* forward (5'-GGTTAACCATGACCGGGAACT-3'), *Cyp1A1* reverse (5'-TGCCCAAAC CAAAGAGAGTGA-3'), *ARNT* forward (5'-CTAAGAGACAGCTTTCAGCAGGT-3'), *ARNT* reverse (5'-AGGGTTTTGGAAGGTAAAGGAG-3').

Plasmids and molecular cloning. Constructs expressing an shRNA against AHR or ARNT were previously generated (Basham *et al.*, 2013) using the pLentiLox5.0-GFP vector (Cai *et al.*, 2007) provided by Dr. James Bear (University of North Carolina, Chapel Hill, NC). Lentiviral expression constructs were generated using the pEiZ plasmid (pHIV-Zsgreen, plasmid 18121, Addgene, Cambridge, MA) previously described (Welm *et al.*, 2008). For generation of pEiZ-HA-AHR, a mouse AHR expression plasmid (pACTAG-HA-AHR) provided by Dr. Oliver Hankinson (University of California, Los Angeles, CA) was used to PCR amplify HA-AHR with the addition of XbaI restriction sites. The following primers were used for amplification:

forward (5'-TAAGCATCTAGAACCATGATCTTTTACCCATACGATGTTCCTG-3'), reverse (5'-TGCTTATCTAGAACCTCAACTCTGCACCTTGCTTAGGAAT-3'). The resulting PCR product was digested with XbaI and ligated into the same site in pEiZ. For generation of pEiZ-HA-AHRR, a mouse AHRR expression plasmid (pcDNA-mAhRR) (Karchner *et al.*, 2002) provided by Dr. Mark Hahn (Woods Hole Oceanographic Institution, Woods Hole, MA) was used to PCR amplify AHRR with addition of an N-terminal HA tag and EcoRI restriction sites. The following primers were used for amplification: forward (5'-CTAGAATTCCCACCATGAG CGTAGTCTGGGACGTCGTATGGGTAATGATGATTCCGTCTGGAGAGTGTACA-3'), reverse (5'-GACGAATTCACTCTAGGGTAGGAAAATTCCATCAGAGCC-3'). The resulting PCR product was subcloned into the pCR®2.1-TOPO® TA vector to create Topo-HA-AHRR, which was then digested with EcoRI, and ligated into the same site in pEiZ. For generation of pEiZ-HA-AHRRmutant, site-directed mutagenesis was performed using the Strategene QuikChange Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) and the Topo-HA-AHRR plasmid according to manufactures instructions. Lys-542, Lys-583, and Lys-660 were each mutated to an arginine residue in separate reactions using the following primer sequences: Lys-542-Arg sense (5'-CACTGGATGTGCCAATCAGGATGGAGAATGAATCTGG-3'), Lys-542-Arg antisense (5'-CCAGATTCATTCTCCATCCTGATTGGCACATCCAGTG-3'), Lys-583-Arg sense (5'-CCAGGATGCACCTGAGAACAGAGCCCGACTA-3'), Lys-583-Arg antisense (5'-TAGTCGGGCTCTGTTCTCAGGTGCATCCTGG-3'), Lys-660-Arg sense (5'-ACTGCAG AGCTCCTATTGTTAGGCGTGAGCCTC-3'), Lys-660-Arg antisense (5'-GAGGCTCACGCC TAACAATAGGAGCTCTGCAGT-3'). Following site-directed mutagenesis, Topo-HA-AHRRmutant was digested with EcoRI and the resulting fragment was ligated into pEiZ using the same site. For generation of pEiZ-HA-ARNT, an HA-tagged mouse ARNT expression

plasmid (pACTAG-HA-ARNT) (Moffett *et al.*, 1997) provided by Dr. Oliver Hankinson (University of California, Los Angeles, CA) was used to PCR amplify the HA-ARNT fragment with additional EcoRI restriction sites. The resulting PCR product was subcloned into the pCR®2.1-TOPO® TA vector to create Topo-HA-ARNT, which was digested with EcoRI and ligated into the same site in pEiZ. The following plasmids for generating lentivirus have been previously described: pMDLg/pRRE (Dull *et al.*, 1998), pRSV-Rev (Dull *et al.*, 1998), and pVSV-G (Clontech, Mountain View, CA). The identity of each plasmid was confirmed by sequencing.

Production and titration of lentivirus. High titer lentivirus was produced, concentrated, and titrated as previously described (Basham *et al.*, 2013).

Generation of stable HC11 cell lines. For stable HC11 cell lines expressing an shRNA or expression construct, $1.25x10^6$ cells were seeded in a 10-cm dish, cultured for 12 hours, and transduced at a multiplicity of infection (MOI) of 20. For expression of HA-AHR, unsorted cells were used for immunofluorescence studies. For all other experiments, transduced cells were sorted 72 hours later based on green fluorescent protein (GFP) expression to obtain stable lines.

RESULTS

AHR agonists inhibit expression of milk genes in mammary epithelial cells- To study the effect of AHR activation on lactation, we sought to recapitulate this process *in vitro*. Initially, we grew primary mammary epithelial cells (MECs) in our three-dimensional branching assay (Basham *et al.*, 2013) in the presence of 1023, an AHR agonist, and performed microarray analysis to determine differentially expressed genes. Compared to vehicle treatment (DMSO), cells grown with 10 μM 1023 showed down regulation of several genes important for milk

production, including *whey acidic protein* and multiple *casein* genes (Table 1). Given the lack of stromal components in this system (Basham *et al.*, 2013; Welm *et al.*, 2008), these results suggested AHR activation blocked milk genes through a direct mechanism in mammary epithelial cells.

To validate these results, we next utilized the HC11 mammary epithelial cell line. HC11 cells were clonally derived from immortalized COMMA-1D epithelial cells (Ball *et al.*, 1988), which were isolated from the mammary gland of midpregnant BALB/c mice (Danielson *et al.*, 1984). Importantly, HC11 cells can be induced to differentiate with lactogenic hormones and produce milk proteins in culture (Ball *et al.*, 1988; Doppler *et al.*, 1990). First, we confirmed that 1023 and TCDD activated AHR in this system. Since AHR translocates to the nucleus following activation (Denison and Nagy, 2003; Heid *et al.*, 2000; Pollenz *et al.*, 1994), we assessed AHR localization following treatment with 1023 or TCDD. Specifically, we stably expressed an HA-tagged AHR construct in HC11 cells and performed immunofluorescence for HA after 24 hours of drug treatment. We found that both 10 μM 1023 and 10 nM TCDD significantly increased the amount of nuclear AHR compared to DMSO (Fig. 1A-B). Furthermore, we observed significant induction of the AHR response gene, cytochrome P4501A1 (*Cyp1a1*) (Whitlock, 1999), in parental HC11 cells following 48 hours of compound treatment (Supplemental Fig. 1A). Together, these experiments demonstrated 1023 and TCDD properly activated the AHR pathway in HC11 cells.

Next, we induced HC11 cells with lactogenic hormones and tested the effect of AHR activation on *β-casein* and *α-casein* expression. Using this assay, cells grown in the presence of 10 μM 1023 or 10 nM TCDD failed to produce the milk protein, β-casein, compared to vehicle treated cells (DMSO) (Fig. 1C). In agreement with our microarray data from primary MECs,

mRNA expression of both *β-casein* and *α-casein* were also inhibited (Fig. 1D and Supplemental Fig. 1B), suggesting a transcriptional mechanism of gene regulation.

Previously, we identified and validated AHR as the biological target of 1023 (Basham *et al.*, 2013). To confirm the observed inhibition of β-casein protein production was dependent on the AHR pathway, we used lentiviral constructs to generate stable HC11 cell lines expressing either a non-specific shRNA (Control) or an shRNA against *AHR* (shAHR #1 and shAHR #2). In cells with ~50% *AHR* knockdown (Supplemental Fig. 1C), we observed a partial rescue in β-casein expression in the presence of AHR agonists (Fig. 1D, Supplemental Fig. 1D). These results suggested 1023 and TCDD inhibited β-casein production in mammary epithelial cells through AHR pathway activation.

The aryl hydrocarbon receptor repressor is sufficient to inhibit β-casein - Given our data in both primary MECs and mammary HC11 cells, we hypothesized AHR activation blocked *β-casein* production through a transcriptional mechanism. Moreover, we became interested in a potential role for AHRR in mediating this process. As a direct target of activated AHR (Mimura *et al.*, 1999), AHRR is highly upregulated in primary MECs treated with 1023 (Basham *et al.*, 2013) and has a known role as a transcriptional repressor. Specifically, AHRR shares high amino acid identity with the N-terminal portion of AHR, which contains both the basic helix-loop-helix (bHLH) and Per-ARNT-Sim "A" (PAS-A) domains (Mimura *et al.*, 1999). These regions of the AHR protein facilitate DNA binding and ARNT dimerization, which allows AHRR to form a heterodimer with ARNT and bind xenobiotic-responsive elements (XREs) in the promoter region of AHR target genes. Subsequent recruitment of a corepressor complex, which includes ANKRA2, HDAC4, and HDAC5, inhibits transcription of AHR target genes (Mimura *et al.*, 1999; Oshima *et al.*, 2007).

To test the effect of AHRR on *β-casein* expression, we first stably expressed a lentiviral HA-tagged AHRR construct in HC11 cells. We confirmed overexpression (Fig. 2A) and validated the construct by measuring a well-characterized AHR response gene, Cyp1A1. Compared to control transduced cells, overexpression of AHRR decreased induction of *Cyp1A1* after 48 hours of treatment with 10 μM 1023 (Fig. 2B). These results verified our tagged AHRR construct interacted with ARNT to functionally repress known target genes.

Next, we tested whether AHRR expression was sufficient to block β-casein production in the absence of AHR agonists. In HC11 cells induced with lactogenic hormones, and without exposure to AHR agonists, β-casein production was repressed at both the mRNA (Fig. 2C) and protein level (Fig. 2D) in cells expressing HA-AHRR compared to control cells. These results demonstrated the presence of AHRR was sufficient to block β-casein expression.

AHRR contains a transcriptional repression domain within its C-terminal region, which consists of three conserved small ubiquitin-like modifier (SUMO)ylation sites at Lys-542, Lys-583, and Lys-660. Previous studies demonstrated these lysine residues are modified by SUMO-1 to facilitate interaction between AHRR and its corepressor complex (Oshima *et al.*, 2009). To genetically test the requirement of this interaction for AHRR to inhibit β-casein production, we mutated all three lysine residues to arginine (HA-AHRRmutant). Previously, these mutations in AHRR have been shown to allow interaction between AHRR and ARNT, but prevent interaction between AHRR and its corepressor complex (Oshima *et al.*, 2009). Compared to HC11 cells overexpressing wild-type AHRR, overexpression of the SUMOylation mutant signficantly rescued β-casein production (Fig. 2E), suggesting AHRR requires interaction with its corepressor complex to inhibit lactogenesis.

ARNT is required for β-casein production in mammary epithelial cells- To elucidate the mechanism through which AHRR inhibits milk production, we examined the role of its major binding partner, ARNT, during normal lactation. Stable HC11 cell lines expressing a nonspecific lentiviral shRNA (Control) or a lentiviral shRNA against ARNT (shARNT #1 or shARNT #2) were generated (Fig. 3A-B). Following induction with lactogenic hormones, knockdown of ARNT inhibited β-casein expression at both the mRNA (Fig. 3C) and protein level (Fig. 3D), and expression of α-casein mRNA (data not shown). As loss of AHR in the absence of any chemical agonists had no effect on β-casein production in HC11 cells (Fig. 1D), these results implicated an independent role for ARNT during milk production.

Overexpression of ARNT rescues β-casein production in the presence of AHR agonists- Based on our results, we hypothesized ARNT participated in a transcriptionally active complex to promote expression of milk genes, such as β-casein, during lactogenesis. Furthermore, since ARNT was required for lactogenesis, we speculated AHRR might inhibit milk production through competitive interactions with ARNT. To test this hypothesis, we overexpressed ARNT prior to treatment with AHR agonists and measured β-casein levels to assess milk production. We reasoned overexpression of ARNT would promote transcriptionally active complexes and restore β-casein levels in the presence of AHR agonists. In HC11 cells stably overexpressing a lentiviral HA-ARNT construct (Fig. 4A), we found β-casein expression partially restored in the presence of both 10 μM 1023 and 10 nM TCDD compared to control cells (Fig. 4B). These results support a model in which AHRR blocks production of β-casein by acting as a transcriptional repressor to inhibit ARNT signaling.

DISCUSSION

Using an epithelial-based model of mammary lactation, we demonstrate AHR agonists directly block β-casein expression. Specifically, we showed AHRR, a robust downstream target of AHR signaling, was sufficient to inhibit the production of β-casein. Although well studied in the context of AHR activation, AHRR is also induced by other toxic insults. In particular, recent studies showed cigarette smoking, including secondhand exposure, caused significant demethylation and increased expression of AHRR (Philibert *et al.*, 2012; Shenker *et al.*, 2013). As women who smoke have consistently been shown to produce significantly less milk volume (Hopkinson *et al.*, 1992; Vio *et al.*, 1991) and breastfeed for a shorter duration (Andersen and Schioler, 1982; Hakansson and Carlsson, 1992; Schulte-Hobein *et al.*, 1992; Vio, Salazar and Infante, 1991; Whichelow, 1979), our results provide a potential molecular mechanism through which this toxicity occurs.

Following induction, AHRR forms a heterodimer complex with ARNT (Evans *et al.*, 2008). As loss of ARNT significantly blocked β-casein production in mammary epithelial cells, our results implicate an important role for ARNT during lactation. These observations are consistent with the phenotype observed in conditional ARNT knockout mice, where ARNT deletion using the MMTV-Cre transgene resulted in impaired mammary function (Le Provost *et al.*, 2002). Specifically, loss of ARNT led to incomplete alveolar development, smaller litter sizes, and 60% of dams that could not support their pups.

However, inactivation of ARNT using WAP-Cre resulted in normal mammary differentiation during pregnancy (Le Provost *et al.*, 2002). Furthermore, transplantation of ARNT null mammary epithelium generated using MMTV-Cre into wild-type recipients normalized alveolar development (Le Provost *et al.*, 2002). Although inconsistent with initial studies and our current findings, these incongruent results may be explained by differences in

methodology. Specifically, WAP-Cre reduced ARNT levels in 80% of the mammary epithelium (Le Provost *et al.*, 2002; Walton *et al.*, 2001), resulting in residual ARNT expression that may facilitate normal development. In reciprocal transplant studies, the high selective pressure of transplantation may have induced compensation from other ARNT family members, including ARNT2. Expressed in the mammary gland (Martinez *et al.*, 2008), ARNT2 is known to form functional complexes with AHR (Hirose *et al.*, 1996) and may be capable of contributing to mammary development. Further *in vivo* studies will be required to explain these differences.

Our experiments with AHRR and ARNT suggest a model in which ARNT promotes milk production under lactogenic conditions (Fig. 5). As a bHLH-PAS family member, ARNT requires dimerization to form a functional transcription complex. Although ARNT is known to interact with multiple proteins, we hypothesize that hypoxia inducible factor-1α (HIF1α) or single-minded 2 (SIM2) interact with ARNT to promote lactation. In previous studies, conditional knockout of ARNT prevented induction of known HIF1α target genes (Tomita *et al.*, 2000), suggesting ARNT is critical for HIF1α signal transduction. Additionally, deletion of HIF1α in the mammary epithelium resulted in severe differentiation defects and failed lactation (Seagroves *et al.*, 2003). Previous studies with SIM2 also support its potential role in cooperating with ARNT to regulate lactation. With SIM2 overexpression, precocious production of β-casein and WAP occurred *in vitro* and *in vivo*, and chromatin immunoprecipitation (ChIP) experiments showed SIM2 associated with the β-casein promoter. Conversely, loss of SIM2 inhibited milk production (Wellberg *et al.*, 2010). Taken together, these experiments strongly implicate a role for HIF1α or SIM2 in promoting ARNT-mediated lactogenesis.

In response to toxic stimuli, our data suggest that AHRR/ARNT heterodimers form and are sufficient to block lactation. One potential mechanism of AHRR repression under these

conditions is ARNT sequestration. Competition for a limited pool of ARNT has been observed previously, where ARNT availability regulated AHR signaling through HIF1α (Gradin *et al.*, 1996) and SIM1 (Probst *et al.*, 1997; Woods and Whitelaw, 2002). However, our experiments using an AHRR repression mutant that could bind ARNT, but not recruit a corepressor complex, suggest this is not the dominant mechanism. Rather, our results favor a model in which AHRR SUMOylation and subsequent recruitment of a corepressor complex that includes ANKRA2, HDAC4, and HDAC5 blocks transcription of genes important for milk production. Further experiments will be needed to determine whether activating ARNT complexes and repressive AHRR/ARNT heterodimers bind the same DNA response elements. Additionally, these studies will help determine whether ARNT-mediated complexes bind directly in the promoter region of milk target genes or whether they control activity of an intermediate factor(s). We have not identified AHR consensus elements within the mouse or human β-casein promoters, and since several milk genes are affected by AHR signaling it is possible global rather than direct pathways attenuate lactation. Further studies will be necessary to determine the targets of AHR signaling that modulate lactogenesis.

Our data demonstrate AHR signaling directly disrupts milk protein production in isolated mammary epithelial cells. Since industrial waste is one of the main sources of TCDD and other related polyhalogenated aromatic hydrocarbons, human exposure to these toxicants is highest in industrialized countries (Schecter *et al.*, 2006a). However, acute exposure has occurred in several distinct populations worldwide, including exposure to Agent Orange herbicide in Vietnam (Schecter *et al.*, 2006b), indigenous Canadian Inuit populations who consume contaminated marine species (Ayotte *et al.*, 1996), and people living in Seveso, Italy during the 1976 industrial explosion (Warner *et al.*, 2002), among others. Given the prevalence of these

environmental pollutants and their ability to bioaccumulate over time, our study has substantial implications on public health, particularly with regard to the ability of women to breastfeed. Thus, future efforts to monitor TCDD exposure levels, analyze epidemiological data, and elucidate the molecular mechanism downstream of AHRR are needed to address this problem.

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FIGURE LEGENDS

FIG. 1. AHR activation blocks milk production in HC11 mammary cells. A, Mammary HC11 cells were transduced with lentivirus to generate a cell line stably expressing HA-tagged AHR. HC11 cells expressing HA-tagged AHR were treated with vehicle control (DMSO), 10 μM 1023, or 10 nM TCDD for 24 hours. Immunofluorescence for HA was performed to measure nuclear translocation of AHR. Dashed white line defines nuclei. Scale bar = 10 μm. B, Quantification of AHR nuclear translocation. An **** indicates a statistically significant difference compared to vehicle treated cells ($p \leq 0.0001$). C, Mammary HC11 cells were treated with vehicle control (DMSO), 10 μM 1023, or 10 nM TCDD and induced with lactogenic hormones (LH) to produce milk proteins. HC11 cell lysates were probed for β-casein and α-tubulin (control). D, *β-casein* mRNA levels were measured in the same assay by RT-PCR and normalized to *β-actin* expression. Results are shown as normalized mean *β-casein* expression (± SEM). An **** indicates a statistically significant difference compared to vehicle treated cells ($p \leq 0.0001$). E, Lentiviral shRNA constructs (shAHR#1 and shAHR#2) were used to stably knockdown *AHR* expression HC11 cells. Stable HC11 cell lines were treated, induced, and probed for β-casein and β-actin (control) as described above. Abbreviations: HA, hemagglutinin; TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; LH, lactogenic hormones; AHR, aryl hydrocarbon receptor.

FIG. 2. AHRR is sufficient to block milk production in HC11 mammary cells. HC11 cells were transduced with lentivirus to generate cell lines stably expressing HA-tagged AHRR (pEiZ-HA-AHRR) or a vector control (pEiZ). A, Western blot of lysates from stable cell lines probed for HA and α-tubulin (control). B, Stable HC11 cell lines were treated for 48 hours with vehicle control (DMSO) or 10 μM 1023. □□□ι□ι mRNA levels were measured by RT-PCR and normalized to *β-actin* expression. Results are shown as normalized mean □□□ι□ι expression (± SEM). An **** indicates a statistically significant difference compared to treated vector control cells ($p \leq 0.0001$). C, Stable HC11 cell lines were induced with lactogenic hormones (LH) to produce milk proteins. *β-casein* mRNA levels were measured by RT-PCR and normalized to *β-actin* expression. Results are shown as normalized mean *β-casein* expression (± SEM). An **** indicates a statistically significant difference compared to treated vector control cells ($p \leq 0.0001$). D, Cell lysates were probed for β-casein and α-tubulin (control). E, An HC11 cell line was generated to express AHRR mutated to prevent SUMOylation in the C-terminal region (HA-AHRRmutant). All stable cell lines were induced with LH and probed for β-casein and α-tubulin (control) as before. Abbreviations: HA, hemagglutinin; AHRR, aryl hydrocarbon receptor repressor; LH, lactogenic hormones.

FIG. 3. ARNT is required for milk production in HC11 mammary cells. A, Lentiviral shRNA constructs (shARNT#1 and shARNT#2) were used to stably knockdown *ARNT* expression in HC11 cells. *ARNT* expression was measured by RT-PCR, normalized to *β-actin* expression, and compared to HC11 cells stably expressing a control shRNA (Control). Results are shown as normalized mean *ARNT* expression (± SEM). An * indicates a statistically significant difference

compared to control shRNA cells ($p \leq 0.05$). B, ARNT protein levels were measured in cell lysates from stable lines. Western blot of lysates probed for ARNT and α-tubulin (control). C, Stable HC11 cell lines were induced with lactogenic hormones (LH) to produce milk proteins. *β-casein* mRNA levels were measured by RT-PCR and normalized to *β-actin* expression. Results are shown as normalized mean *β-casein* expression (± SEM). An **** indicates a statistically significant difference compared to treated control cells ($p \leq 0.0001$). D, Western blot of lysates probed for β-casein and α-tubulin (control). Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; LH, lactogenic hormones.

FIG. 4. ARNT overexpression rescues milk production in the presence of AHR agonists. A, A HA-tagged ARNT construct (pEiZ-HA-ARNT) was stably expressed in mammary HC11 cells using lentiviral transduction. An empty expression vector (pEiZ) was used to create a stable control HC11 cell line. Western blot of lysates probed for HA and α-tubulin (control). B, Stable HC11 cell lines were treated with vehicle control (DMSO), 10 μM 1023, or 10 nM TCDD and induced with lactogenic hormones (LH) to produce milk proteins. Western blot of lysates probed for β-casein and α-tubulin (control). Abbreviations: HA, hemagglutinin; ARNT, aryl hydrocarbon receptor nuclear translocator; LH, lactogenic hormones; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

FIG. 5. Working model for the mechanism through which AHR activation blocks milk production in mammary epithelial cells. (Right) In the absence of AHR agonists, we propose ARNT signaling promotes milk production during lactogenic stimulation. (Left) In the presence of AHR agonists, AHR translocates to the nucleus and forms a heterodimer with ARNT.

AHR/ARNT heterodimers bind XRE sequences to increase transcription of AHR target genes, including AHRR. Once induced, AHRR competes with AHR for interaction with ARNT and forms a transcriptionally repressive complex, which we propose inhibits expression of milk proteins. Dashed lines indicate aspects of the model proposed by the current data.

Table 1. Genes involved in milk production down regulated in MECs treated for 72 hours with 10 μM 1023

Gene identifier	Gene name	Gene symbol	Ratio*
NM_007784	Casein alpha s1	*Csn1*	-10.97
NM_007786	Casein kappa	*Csn3*	-8.24
NM_011709	Whey acidic protein	*Wap*	-7.88
NM_009972	Casein beta	*Csn2*	-6.84

**Log2 ratio for 1023-treated versus DMSO*